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USAF CELLULAR MECHANISM OF TURNOVER OF THE STRESS INDUCED PROTEIN HSP70.

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6. AUTHOR(S)

Dr Nancy S. Petersen

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dept of Molecular Biology
P.O. Box 3944
Univ of Wyoming
Laramie, WY 82071

9. SPONSORING MONITORING AGENCY NAME(S) AND ADDRESS(ES)

AFOSR/NL
110 DUNCAN AVE SUITE B115
BOLLING AFB DC 20332-0001

Dr Walter Kozumbo

AFOSR-TR- 94 0480

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13. ABSTRACT (Maximum 200 words)

Because heat shock proteins are made by all organisms in response to environmental stress, it has been proposed that accumulation of these proteins could be useful in environmental monitoring. In order to use the accumulation of heat shock proteins as indicators of environmental stress, it is important to understand how their stability is regulated. This research is concerned with determining the influences that regulate the stability of the major heat shock protein, hsp70, in rainbow trout (used for environmental monitoring) and in fruit files (a well characterized system used for basic research). During the tenure of this grant progress has been made characterizing the rainbow trout heat shock response, cloning and sequencing the rainbow trout heat shock gene, and in generating antibodies specific for fruit fly and rainbow trout hsp70. The accumulation of hsp70 in juvenile rainbow trout exposed to heaby metals has been assessed in collaboration with the H. Berman Lab. Commercially available antibodies have been used to identify hsp70 breakdown products in flies, trout, chick and mouse, and the sequences the major breakdown fragments of the fly hsp70 generated in vivo have been determined.

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USAF Cellular Mechanism of Turnover of the Stress Induced Protein HSP70.

Dr. Nancy S. Petersen
Department of Molecular Biology
P.O. Box 3944
University of Wyoming
LAramie, Wyoming 82071

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USAF Cellular Mechanism of Turnover of the Stress Induced Protein HSP70.

ABSTRACT

Because heat shock proteins are made by all organisms in response to environmental stress, it has been proposed that accumulation of these proteins could be useful in environmental monitoring. In order to use the accumulation of heat shock proteins as indicators of environmental stress, it is important to understand how their stability is regulated. This research is concerned with determining the influences that regulate the stability of the major heat shock protein, hsp70, in rainbow trout (used for environmental monitoring) and in fruit flies (a well characterized system used for basic research). During the tenure of this grant progress has been made characterizing the rainbow trout heat shock response, cloning and sequencing the rainbow trout heat shock gene, and in generating antibodies specific for fruit fly and rainbow trout hsp70. The accumulation of hsp70 in juvenile rainbow trout exposed to heavy metals has been assessed in collaboration with the H. Berman lab. Commercially available antibodies have been used to identify hsp70 breakdown products in flies, trout, chick and mouse, and the sequences the major breakdown fragments of the fly hsp70 generated *in vivo* have been determined.

a. Summary of Objectives

Summary:

Synthesis of the heat shock protein, hsp70, appears to be essential for recovery from heat and chemical stress. Both because of the role of this protein in cellular recovery from stress and because of the possibility of using levels of hsp70 synthesis or accumulation as a measurement of cellular response to stress, it is important to study the stability of hsp70.

We have shown that Drosophila hsp70 decays *in vitro* by an autoproteolytic mechanism (Mitchell et al., 1985). Autoproteolytic decay could be part of the feedback mechanism regulating the levels of hsp70 accumulation if it occurs *in vivo*. To determine whether autoproteolytic decay is occurring *in vivo*, we propose to identify the *in vivo* breakdown products of hsp70 and to compare their N-

terminal sequences to those of the *in vitro* breakdown products. Precisely the same cutting site would indicate that the same protease may be responsible for the decay in both cases. We will also determine the site of the protease activity in hsp70 for the *in vitro* decay.

Autoproteolytic decay is part of the normal processing which occurs in many viral proteins and leads to the production of functional proteins. Perhaps breakdown products of hsp70 also have a function in the cell. In any case, if one of them is more stable than hsp70 itself, it may be more useful than intact hsp70 as a measure of cellular exposure to stress. An initial step toward looking at these possibilities is to identify the *in vivo* breakdown products of hsp70 and look at the stability of both hsp70 and its decay products *in vivo*. We propose to do this in Drosophila larvae and rainbow trout tissues.

During the tenure of this grant we propose to:

1) Identify the *in vivo* breakdown products of Drosophila and rainbow trout hsp70.
2) Sequence the N-termini of both the *in vivo* and the *in vitro* decay products of Drosophila and rainbow trout hsp70.
3) Identify the sites of proteolytic activity in both rainbow trout and Drosophila hsp70.
4) Use antibodies to hsp70 to look in detail at the kinetics of synthesis and decay of hsp70 and its breakdown products in Drosophila larvae and rainbow trout tissues.

b. Research Progress

Progress Report:

This proposal was originally submitted in April of 1989. By the time it was funded in April of 1992, several changes in the research plan had been negotiated with Lt. Col. T. Jan Cerveny. The most important of these was the substitution of rainbow trout for rats as the second model system to be studied. (The other model system is Drosophila.) This change necessitated the cloning and sequencing of the major trout hsp70 gene, since only partial sequences of the most

conserved region of the hsp70 gene have been published for rainbow trout. We also added experiments to directly test the usefulness of hsp70 as a biomarker as part of a collaborative experiment with Harold Bergman's group which does water quality work at the University of Wyoming.

The goals of this project are to develop antibodies to the N-terminal and C-terminal parts of hsp70 in Drosophila and rainbow trout, and to use these antibodies to identify and characterize the major breakdown products of hsp70. The construction of expression vectors was proposed in order to produce large quantities of both the human and the Drosophila hsp70 in *E. coli*. Production of the protein in *E. coli* has the advantages that large quantities of protein can be made, and that it will not be necessary to separate hsp70 from its non-heat-inducible cellular homologues (called cognates). The protein made in *E. coli* would be used for two purposes. First, to generate polyclonal antibodies in rabbits and rats. These antibodies would provide specific reagents to recognize the major decay products of hsp70 and they would be used to identify and to isolate the decay products from Drosophila cells and rainbow trout. Second, the hsp70 protein produced in vitro would be used to generate large quantities of decay products so that the N-termini of the in vivo *and in vitro decay* products could be sequenced and compared. An autoproteolytic mechanism was proposed for decay of hsp70 *in vitro*. Purification and sequencing of the N-termini of both the *in vivo* and the *in vitro* decay products was proposed to see whether identical digestion products are produced *in vivo* and *in vitro*.

Rainbow trout heat shock response:

The heat shock response has been characterized in rainbow trout liver and gill tissue. These tissues were dissected, heated, and labeled with ^{35}S-methionine immediately after euthanizing the trout. The labeled proteins were visualized on autoradiograms of SDS-polyacrylamide gels. The increased synthesis of heat shock proteins is first detected at 20°C. The maximum synthesis of hsp70 occurs at at different temperatures in different tissues. Maximum heat shock protein synthesis in liver and brain occurs at 24°C while in gill and kidney maximum synthesis is at 28°C. Protein synthesis greatly inhibited in all of the tissues at 30°C.

Initial experiments looked at the decay of hsp70 made during a one

hour heat treatment in rainbow trout liver (26°C) and gill (28°C). Decay was followed at 18°C and 10°C. Decay was most rapid in gill at 18°C. Under these conditions all of the labeled hsp70 had disappeared by 48 hours. In liver, decay of hsp70 was detectable by three days, but a half life was not determined due to difficulty keeping tissue alive for a longer period of time. No decay was detectable during three days at 10° in either tissue.

In order to express the rainbow trout gene in *E.coli* and obtain antibodies to the rainbow trout hsp70, we have isolated mRNA and used PCR to obtain cDNA for cloning. Messenger RNA was isolated from liver tissue which was kept at 10°C or at 26°C in RPMI 1640 tissue culture media (Gibco) for an hour before freezing and mRNA preparation. PCR on reverse transcribed mRNA shows one mRNA band present in both control and heat shocked samples. PCR primers were designed to conserved regions at the N-terminus and C-terminus of the coding region and amplify DNA of the predicted size of 1.8 kb. The PCR product has been cloned in to Bluescript and several clones are ready for sequencing. We are in the process of sequncing the major trout hsp70 gene. The sequence we have to date is 67% homologous to the middle part of the carboxy-terminal Drosophila sequnce and appears below:

```
        10         20         30         40         50
         *          *          *          *          *
TCTACTAATA CGCAGGGTGT AGCCGAGGTC ACCATGACGC CCGAAAGAAA

        60         70         80         90        100
         *          *          *          *          *
CGGTTCGTAT ATGGTGAAAG CATCCCTGCC GAATGGAGCC TCACTTGAGA

       110        120        130        140        150
         *          *          *          *          *
AACAACTGGA GGCTATTGAT GAAAAACTGA CACTCACGGC GTCCAGTCCG

       160        170        180        190        200
         *          *          *          *          *
CTTATCGGTG TCTATGCCCC TACAGGCGCT ACTCTGACGG CAACGACTAA

       210        220        230        240        250
         *          *          *          *          *
CCTCTGCAAA TGGCACTCCA GTGGAGGTCA GGTCATCAAC TTTAGCGTAC

       260        270
         *          *
GCCAGAAGGC GTACCGATTA GTGCGAAAG
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Use of antibodies to detect changes in hsc70 (heat shock cognate) levels in trout tissues after exposure to metal contaminated water:

Rainbow trout were exposed for three weeks to clean water or water with two times the EPA allowable concentrations of Zn^{++}, Cu^{++}, Cd^{++}, and Pb^{++} (100µg/L Zn^{++}, 24µg/L Cu^{++}, 2.2µg/L Cd^{++}, and 6.4µg/L Pb^{++}). Trout were pithed (adults) or decapitated (juveniles). Tissues (gill, liver, kidney, stomach, pyloric ceceum) were labeled with ^{35}S-methionine. In all 24 adult rainbow trout, and 24 juvenile trout were examined. Proteins were extracted from each sample and run on SDS-PAGE gels. The gels were blotted to PVDF paper and StressGen 72/73 antibody to hsp70 was used to detect levels of hsp70 related proteins. Blotting conditions were worked out so that the staining of hsp70 increased linearly with protein concentration. There were very different ^{35}S-methionine labeling patterns for the different tissues, however exposure to metal contaminated water did not show detectable increases in hsp70 synthesis or synthesis of any other major proteins seen on these autoradiograms. The levels of hsp70 in the different tissues were quite variable, but were not significantly different between controls and exposed trout with one exception. Juvenile gills from trout raised in 2X water and contaminated food have at least two times the amount of hsp70 as the controls. One problem we encountered was that the Stress-Gen antibody reacted only weakly with the rainbow trout tissues as compared to mouse and chick tissues. This could be due to lower levels of hsp70 in trout or to lower affinity of the antibody for trout hsp70. We will be able to obtain a more specific antibody using the trout hsp70 gene expressed in *E. coli*.

Antibodies to Drosophila hsp70 fragments:

We have subcloned three different fragments of hsp70 into the PGEX expression vectors. These fragments represent the conserved amino terminus of the protein, the carboxy-terminus of the protein, and an internal sequence (the Ava1 - BamH1 fragment) which has been implicated in autoproteolysis. Each fragment was expressed in *E. coli* as a glutathione S-transferase fusion protein. The fusion proteins were purified by binding to glutathione sepharose beads. Purification of the hsp70 fragments was done by thrombin digestion at a thrombin site engineered at the junction between the two sequences.

Antibodies to each fragment have made in rat by Cocalico Biologicals Inc., Reamstown, PA. Interestingly, the antibody to the Drosophila caboxyterminal portion of hsp70 reacts much more strongly with fish hsp70 than the commercially available antibodies to human and Drosophila hsp70.

Turnover of hsp70 in Drosophila tissues:

Our earlier experiments and those of Karen Palter and Elizabeth Craig (1986) had shown that hsp70 in whole larvae and adult flies has a half life of 3-5 hours. We have repeated these experiments using individual tissues from larvae and pupae rather than whole animals. We wanted to see if there were major differences in the half life of hsp70 in tissues and whether the the breakdown was a cellular event or whether it required interaction of tissues in the whole animal. Brains and salivary glands were dissected from 3rd instar larvae. Wings were dissected from pupae of different ages. These were labeled with 35S-methionine during a mild heat shock of 35° for 30 min., and chased for 2- 6 hours at 25°C. Hsp70 decays with a half life of 3-4 hours in different larval and pupal tissues. The half life of hsp70 in wings was the same at different developmental stages. These results indicate that the turnover of hsp70 occurs at the cellular level and is similar in the different tissues and at the different times which were examined.

More experiments comparing the turnover of hsp70 and that of hsc70 in Drosophila show that the turnover of these proteins depends on the heat shock and recovery conditions. Hsc70 made at 25°C is stable for at least six hours during a chase at 25°C. However, hsc70 made at 35°C decays with the same half life as hsp70 when it is shifted to 25°C. These experiments lead us to suggest that the decay of hsp70 is regulated to allow the return to normal levels of hsp/hsc70 following heat shock.

Breakdown Products of hsp70

Breakdown products of hsp70 have tentatively been identified in tissues from rainbow trout, Drosophila, mouse, and chicken. We have used three commercially available monoclonal antibodies to look for potential breakdown products of hsp70 or hsc70 in heat shocked trout liver, mouse liver, chicken liver, and Drosophila salivary glands. The antibodies used were StressGen SPA-820 and Affinity Bioreagents MA3-006 and MA3-007.

All three antibodies reacted strongly with a 70kD band in mouse and chick. In mouse there was also a major cross reacting band at 46kD (the approximate size expected for the breakdown product). In chicken cross reacting bands at 44kD and 36kD were observed. The Affinity Bioreagents antibodies reacted weakly to Drosophila and trout 70kD proteins. In Drosophila, MA3-007 (to a conserved epitope in the N-terminus of the protein) reacted with a band at 43kD, indicating that there may be a 43kD breakdown product in Drosophila salivary glands, and that if so, it is derived from the N-terminal region of the protein.

The StressGen antibody reacted weakly with trout and not at all with Drosophila hsp/hsc 70. Since it is a monoclonal, it will recognize only breakdown products which include the one epitope it recognizes. Breakdown products identified using the StressGen antibody in trout have molecular weights of 36kD and 34kD. The amount of each of these varies in different tissues.

We have isolated the *in vitro* breakdown products of Drosophila hsp70 from gels and N-terminal sequence analysis indicates that the position of the two major cutting sites are after amino acids 68, and 260 respectively. The sequences surrounding these cut sites are AK^RLI and LR^RLR. These sites are among the most highly conserved regions in this protein, indicating that a structural feature in this region or the mechanism of breakdown itself is important to the function of this protein.

c. Publications

(1992) Petersen, N. S., Williams, J., and Young, P. Hsp70 is an Unstable Protein in Drosophila Tissues and Cell Lines. Molecular Biology of the Cell <u>3</u>; s1031.

Papers in preparation:

Williams, J., Young, P., Farag, A., Bergman, H., and Petersen, N. Increased levels of hsp70 in juvenile rainbow trout gills exposed to heavy metals.

Petersen, N., Young, P., and Williams, J. Regulation of the turnover of hsp70 and hsc70 in Drosophila tissues.

Williams, J., Young, P., and Petersen, N. The heat shock response in juvenile rainbow trout.

d. Personnel

Nancy S. Petersen	P. I.
Pat Young	Research Associate
Jeanne Willliams	Graduate Student
Audrey Hayes	Undergraduate, lab assist.
	Undergraduate, fly care

e. Papers Presented at Meetings.

(1992) Petersen, N.S., Williams, J., and Young, P. Rapid Turnover of hsp70 in Drosophila larval and pupal tissues. ASCB Molecular Chaperonins Meeting Abstracts.

(1992) Petersen, N. S., Williams, J., and Young, P. Hsp70 Stability, SETAC News, 13th Annual Meeting Abstracts.

(1992) Williams, J.H., Farag, A.M., Stansbury, M.A., Young, P.A., Petersen, N.S., and Bergman, H.L. Biochemical and physiological differences observed in rainbow trout fed different diets. SETAC News, 13th Annual Meeting Abstracts.

(1992) Farag, A., Williams, M.A., Stansbury, M.A., Beose, C.J., Young, P.A., and Bergman, H.L. Milltown Reservoir - Clark Fork River, Montana: The physiological responses of rainbow trout exposed to metals in the water and diet. SETAC News, 13th Annual Meeting Abstracts.